

# CTX-M enzymes: origin and diffusion

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CTX-M  $\beta$ -lactamases are considered a paradigm in the evolution of a resistance mechanism. Incorporation of different chromosomal *bla*<sub>CTX-M</sub> related genes from different species of *Kluyvera* has derived in different CTX-M clusters. *In silico* analyses have shown that this event has occurred at least nine times; in CTX-M-1 cluster (3), CTX-M-2 and CTX-M-9 clusters (2 each), and CTX-M-8 and CTX-M-25 clusters (1 each). This has been mainly produced by the participation of genetic mobilization units such as insertion sequences (*ISEcp1* or *ISCR1*) and the later incorporation in hierarchical structures associated with multifaceted genetic structures including complex class 1 integrons and transposons. The capture of these *bla*<sub>CTX-M</sub> genes from the environment by highly mobilizable structures could have been a random event. Moreover, after incorporation within these structures,  $\beta$ -lactam selective force such as that exerted by cefotaxime and ceftazidime has fueled mutational events underscoring diversification of different clusters. Nevertheless, more variants of CTX-M enzymes, including those not inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid (IR-CTX-M variants), only obtained under *in vitro* experiments, are still waiting to emerge in the clinical setting. Penetration and the later global spread of CTX-M producing organisms have been produced with the participation of the so-called “epidemic resistance plasmids” often carried in multi-drug resistant and virulent high-risk clones. All these facts but also the incorporation and co-selection of emerging resistance determinants within CTX-M producing bacteria, such as those encoding carbapenemases, depict the currently complex pandemic scenario of multi-drug resistant isolates.

**Keywords:** *bla*<sub>CTX-M</sub> genes, *Kluyvera* spp., gene mobilization, *ISEcp1*, *ISCR1*, plasmid, bacterial clones, antibiotic selective force

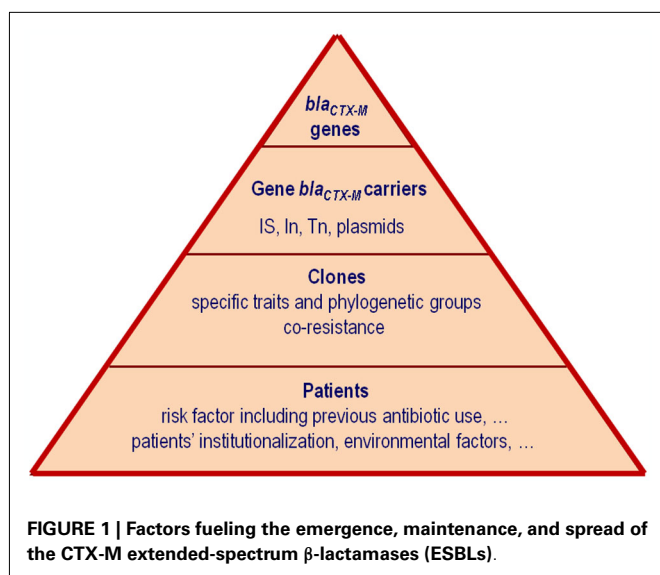
## INTRODUCTION

Antibiotic resistance is a topic in continuous review and the several studies published every year with new data can modify or redefine our perspective about this problem.  $\beta$ -lactam resistance and particularly overviews about  $\beta$ -lactamases, enzymes able to hydrolyze  $\beta$ -lactam antibiotics, have undoubtedly been the most frequently studied topics in this field.  $\beta$ -lactams, the antibiotics most widely used all over the world, have also given rise to a continuous increase of resistant isolates due to their permanent selective force driving diversification of the resistance mechanisms.

$\beta$ -Lactamases have been studied from many different aspects, including classification, hydrolytic profiles and kinetic properties, plasticity, frequency, and dissemination. Moreover they have been used as an example of the accelerated evolution following the Darwinian paradigm, where the strong antibiotic selective pressure favors the survival of the most adapted. According to a recent review from Davies and Davies (2010), a dramatic increase in the number of  $\beta$ -lactamases has been described since the 1980s of the last century but this increase is due almost exclusively to class A and D  $\beta$ -lactamases (Bush and Jacoby, 2010). Among the class A  $\beta$ -lactamases, the extended-spectrum  $\beta$ -lactamases (ESBLs) which are able to hydrolyze expanded-spectrum cephalosporins (such as cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam) represent

a public health concern (Coque et al., 2008a; Pitout and Laupland, 2008). ESBLs of class A mainly include TEM, SHV, CTX-M, VEB, and GES enzymes. Among them, the highest number of variants described in the last years corresponds to the CTX-M family (123 variants until 2011, last accession December 5, 2011)<sup>1</sup>. This explosive dissemination of CTX-Ms around the world has been referred as the “CTX-M pandemic” due to their increasing description worldwide (Cantón and Coque, 2006). Although different updates about CTX-M  $\beta$ -lactamases have been published (Bonnet, 2004; Livermore et al., 2007; Rossolini et al., 2008; Hawkey and Jones, 2009; Naseer and Sundsfjord, 2011), new data about dispersion and clonality of CTX-M producing isolates, molecular epidemiology, protein plasticity, evolution and origin of the *bla*<sub>CTX-M</sub> genes, influence of antibiotic use, and patients risk factors, justify this new review. **Figure 1** illustrates different factors fueling the emergence, maintenance, and spread of the CTX-M ESBLs. We will focus our attention on the epidemiology, origin and evolution of *bla*<sub>CTX-M</sub> genes, and influence of surrounding genetic structures participating in the maintenance and spread of these genes and hence organisms producing CTX-M enzymes.

<sup>1</sup> [www.lahey.org/Studies/other.asp](http://www.lahey.org/Studies/other.asp)



## CLASSIFICATION OF $\beta$ -LACTAMASES AND CTX-M ENZYMES

$\beta$ -Lactamases are by far the most important resistant mechanisms in Gram-negative bacilli. With the popularization of molecular techniques, an increasing number of these enzymes have been characterized differing in amino acid sequences and hydrolytic activity against  $\beta$ -lactam antibiotics (Bush, 2010). They are mainly classified using the Ambler scheme (Ambler, 1980), which grades to the so-called molecular classes (A through D) and also currently functional groups which use the characteristic of the enzymes, including their hydrolytic substrate profile, and response to inhibitors (Bush and Jacoby, 2010). Classically, genetic location of the corresponding  $\beta$ -lactamase genes (*bla*) in plasmids or chromosome was also used to differentiate these enzymes. Nevertheless, this genetic characteristic is no longer used, as chromosomal *bla* genes can be mobilized and integrated into plasmids or transposons, but a reverse situation of initially describes plasmid-mediated  $\beta$ -lactamases into the chromosome is also increasingly found (Toleman et al., 2006; Coelho et al., 2010). In addition protein regulation characteristics (i.e., constitutive or inducible expression) are also noted when referring to different  $\beta$ -lactamases groups (Livermore, 1995) but this trait depends on the surrounding genes. In Gram-negative organisms, inducible expression of  $\beta$ -lactamases is commonly found in chromosomal  $\beta$ -lactamases whereas plasmid-mediated enzymes are generally constitutively expressed. Enhancement expression of their hydrolytic activity is often regulated by promoters present in upstream genes.

Within the  $\beta$ -lactamases, the ESBLs have been worthy of the attention of the scientific community and clinicians over the last three decades since their first description in 1983 (Knothe et al., 1983). In general, ESBLs are located in plasmids and are characterized for their ability to hydrolyze oxy-imino-cephalosporins (third and fourth generation cephalosporins) and monobactams but not cephamycins such as cefoxitin and carbapenems including imipenem, ertapenem, meropenem, or doripenem (Philippon et al., 1989; Jacoby and Muñoz-Price, 2005; Bush and Fisher,

2011). In addition, they are generally susceptible to  $\beta$ -lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Mainly, they are included in the 2be Bush-Jacoby and Medeiros functional group belonging to the molecular class A  $\beta$ -lactamases from the Ambler's classification (Ambler, 1980; Bush et al., 1995; Bush and Jacoby, 2010). Enzymes showing an "ESBL phenotype" have been mainly described in TEM-, SHV-, CTX-M, GES, and VEB families into class A  $\beta$ -lactamases, and OXA-ESBLs into class D  $\beta$ -lactamases (Paterson and Bonomo, 2005).

The TEM-, SHV-, and OXA-type ESBL enzymes derive from point mutations from the classical plasmid-mediated enzymes TEM-1, TEM-2, SHV-1, and OXA-10 which are not of greater importance in hydrolyzing extended-spectrum cephalosporins (Paterson and Bonomo, 2005; Bush and Jacoby, 2010). The TEM- and SHV-ESBLs were predominant in the ESBL landscape over the 1980s and 1990s in the past century, mainly associated with outbreaks in hospitals involving *Klebsiella pneumoniae* and to a lesser extent in *Escherichia coli* and other Enterobacteriaceae whereas the CTX-M were less prevalent (Bradford, 2001; Paterson and Bonomo, 2005). Although first discovered in 1989 (Bauernfeind et al., 1990, 1992), the CTX-M enzymes did not reach prominence over the other ESBL enzymes until the first decade of the 2000s when accelerated evolution and extraordinary dispersion of these enzymes were observed (Cantón, 2008). They were confined not only to the hospital setting but also to the community, with *E. coli* being the most important pathogen producing these enzymes (Cantón and Coque, 2006; Coque et al., 2008a).

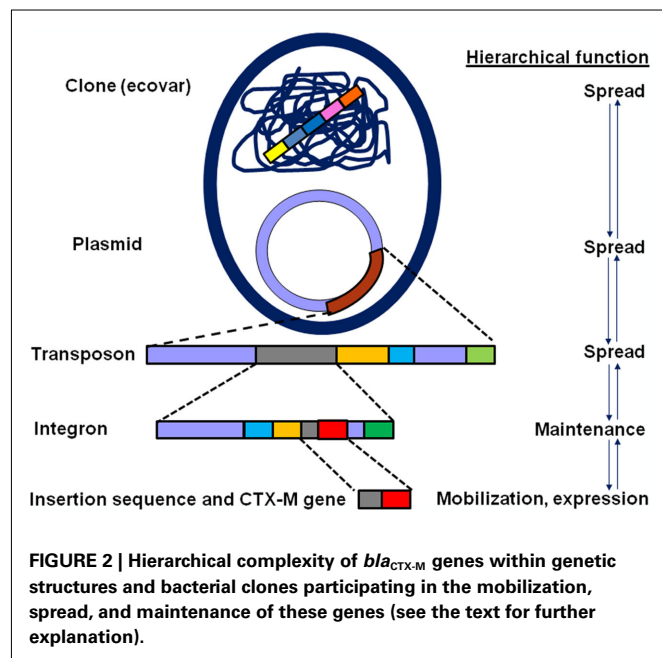
Unlike other ESBLs, CTX-M family constitutes a complex and non-homogeneous group of enzymes. The first view and alignment of the amino acid sequence of the different CTX-M variants allowed to classify these enzymes in five clusters but recent publications report at least two more additional clusters (Bonnet, 2004; Rossolini et al., 2008; see also below). Phylogenetic analyses suggest that CTX-Ms were not originated by mutations from previous plasmid mediated enzymes but through mobilization of chromosomal *bla* genes from *Kluyvera* spp. when they were incorporate into mobile genetic elements (Cantón, 2008). These original mobilized *bla*<sub>CTX-Ms</sub> genes affected cefotaxime to a higher degree than ceftazidime. Nevertheless and from an evolutionary point of view, CTX-Ms as other ESBLs later diverged by punctual mutations probably as consequence of antibiotic selective pressure once *Kluyvera* spp. *bla*<sub>CTX-M</sub> genes were mobilized and were incorporated into mobile genetic elements. This also gave the CTX-M enzymes the opportunity to enhance the hydrolytic activity against ceftazidime and a new variants were described (Bonnet, 2004; Poirel et al., 2008).

## EPIDEMIOLOGY OF CTX-M $\beta$ -LACTAMASE-PRODUCING BACTERIA

Studies over the last 10 years have revealed that unlike some exceptions, the CTX-M enzymes have nearly displaced other ESBLs enzymes in Enterobacteriaceae, including TEM and SHV ESBL variants (Cantón, 2008; Coque et al., 2008a; Angel Díaz et al., 2009; Hawkey and Jones, 2009; Bush, 2010; Rodríguez-Villalobos et al., 2011). This displacement might have occurred not only as a consequence of the extraordinary dissemination of the corresponding

*bla*<sub>CTX-M</sub> genes in highly mobilizable genetic platforms, including plasmids and transposons, but also because of these platforms within successful clones (Cantón and Coque, 2006; Rogers et al., 2011; Woodford et al., 2011; **Figure 2**). Another reason for this increase is the co-resistance phenomenon in CTX-M producing organisms, particularly to aminoglycosides and fluoroquinolones, which might facilitate co-selection processes (Morosini et al., 2006; Cantón and Ruiz-Garbajosa, 2011).

Apart from this general overview, within the CTX-M enzymes, the CTX-M-15, and CTX-M-14 are by far the most important ones, virtually invading all human and animal compartments as well as the environment all over the world (Cantón et al., 2008; Hawkey and Jones, 2009; Dolejska et al., 2011; Hiroi et al., 2012). Nevertheless, temporal emergence and penetration of these enzymes in different epidemiological scenarios might also explain the current epidemiology of CTX-M enzymes. Antibiotic consumption and dissimilar risk factors in different geographic areas and groups of patients and particularities of different compartments might have also contributed to the current CTX-M scenario (Carattoli, 2008; Rodríguez-Baño and Navarro, 2008; Rodríguez-Baño and Pascual, 2008; Oteo et al., 2010a; Naseer and Sundsfjord, 2011). In this sense three different periods of CTX-M can be differentiated. The first one includes emergence of different CTX-M  $\beta$ -lactamases in different and distant geographic areas and might have occurred until the mid-1990s of the last century. The second was characterized by the emergence of the most widespread CTX-M enzymes, including CTX-M-3, CTX-M-9, CTX-M-14, and CTX-M-15 enzymes and might have occurred from 1994 to 2000. Some of these enzymes, but not exclusively, are variants of previously described CTX-M  $\beta$ -lactamases. Finally, the third period through 2000 is characterized by the universal dispersion and globalization of these  $\beta$ -lactamases. The second and third periods will be presented together below.



## THE EMERGENCE OF CTX-M $\beta$ -LACTAMASES: DIFFERENT CTX-M ENZYMES IN DIFFERENT COUNTRIES

The first recognition of the emergence of CTX-M  $\beta$ -lactamases occurred nearly simultaneously in Europe and South America at the beginning of 1989 (Bauernfeind et al., 1990, 1992). The first publication recognizing an ESBL from the CTX-M group was that reporting a cefotaxime-resistant but ceftazidime susceptible *E. coli* isolate recovered from the ear of a 4-month-old child suffering from otitis media in Munich (Germany; Bauernfeind et al., 1990). The enzyme responsible for this particular ESBL phenotype not affecting ceftazidime was named as CTX-M-1 in reference to its preferential hydrolytic activity against cefotaxime (CTX as its acronym, -M from Munich). This enzyme inaugurated a group of enzymes that during the 1990s were also grouped as cefotaximases due to their particular phenotypic and hydrolytic profile (Bonnet, 2004). In South America, the first isolates with a “cefotaximase pattern” were detected in *Salmonella typhimurium* recovered in hospitalized patients suffering from meningitis, septicemia, or enteritis (Bauernfeind et al., 1992). The enzyme responsible for this phenotype had a different isoelectric point (pI 7.9) than the one (pI 8.9) described in Germany and was named as CTX-M-2. Later sequencing of *bla*<sub>CTX-M-2</sub> gene in 1996 confirmed a different amino acid sequence of these two enzymes but a shared homology of 84% (Bauernfeind et al., 1996).

These CTX-M-1 and CTX-M-2  $\beta$ -lactamases were the first reported CTX-M like enzymes but not chronologically the enzymes belonging to this group. They were preceded by the isolation in 1986 of a cefotaxime-resistant *E. coli* isolate recovered from the fecal flora of a laboratory dog which was used for pharmacokinetic studies of  $\beta$ -lactam antibiotics in Japan (Matsumoto et al., 1988). The enzyme responsible for this phenotype was named as FEC-1 (Fecal *E. coli*), which was later recognized to be related with the CTX-M-3 enzyme initially found in Poland (Bonnet, 2004). Moreover, and also at the beginning of 1989 but in France, an *E. coli* with an identical phenotype was isolated from a Italian patient (Barthélémy et al., 1992; Bernard et al., 1992). The  $\beta$ -lactamase, named as MEN-1 (named after the patient in who it was isolated), was the first available sequence of a CTX-M  $\beta$ -lactamase (Barthélémy et al., 1992). In 1996 sequencing of both the original CTX-M enzymes, CTX-M-1 and CTX-M-2, revealed that MEN-1 was identical to CTX-M-1 (Bauernfeind et al., 1996).

Other CTX-M related  $\beta$ -lactamases that were contemporarily isolated during the 1990s were Toho-1 and Toho-2 enzymes (Toho refers to Toho University School of Medicine Omori Hospital in Tokyo). The former were characterized in a cefotaxime-resistant *E. coli* isolate recovered in 1993 from a 1-year-old female child who developed cystitis in Japan (Ishii et al., 1995) and the latter also in a cefotaxime-resistant *E. coli* isolate recovered in Japan in 1995 from the urine of a 69-year-old male who suffered from colon cancer (Ma et al., 1998). Sequence alignment revealed their linkage with CTX-M-2. Both enzymes were later renamed and designated as CTX-M-44 (Toho-1) and CTX-M-45 (Toho-2; last accession December 5, 2011)<sup>2</sup>

<sup>2</sup><http://www.lahey.org/Studies/>.

All these earliest descriptions of CTX-M enzymes showed hot areas for their emergence (Central Europe, South America, and Japan) that could reflect not only specific selective driving forces such as antibiotic consumption but also geographic specificity for different enzymes that could be related with their presence in specific clones or abundance of organism from which they derived (see below). Unfortunately, clonality of ancestral CTX-M producing isolates was not performed as well as plasmid characterization. These studies would have been relevant for tracing the emergence and dissemination of the first CTX-M ESBLs. Moreover, epidemiological surveys including molecular characterization at the end of the 1980s from other geographic areas, such as South East Asia that is nowadays relevant for emerging  $\beta$ -lactamases, including carbapenemases (enzymes that are able to hydrolyze carbapenems), were absent. These would also have been important for the better understanding of the current dissemination of epidemiologically important CTX-Ms that were described through 1994, such as CTX-M-3, CTX-M-9, CTX-M-14, and CTX-15. **Table 1** includes epidemiological data of the earliest descriptions of different CTX-M ESBLs during the 1980s and 1990s. This data are relevant for understanding current epidemiological scenario.

#### PENETRATION AND GLOBALIZATION OF CTX-M ENZYMES ALL OVER THE WORLD

During the 1990s, different reports showed the appearance nearly at the same time of the same enzymes in very distant countries, denoting the potential independent emergence of these enzymes but also suggesting a rapid dissemination. During these years, diversification was also noted with the ever increasing description of new CTX-M enzymes from different groups. This has been well illustrated with CTX-M-3, a closely related enzyme with CTX-M-1 differing in four amino acid positions (V77A, D114N, S140A, and N288D). This enzyme was detected in *E. coli* and *K. pneumoniae* isolates recovered in 1996–1997 in Poland (Gniadkowski et al., 1998; Palucha et al., 1999), in 1998 in France (De Champs et al., 2000; Doucet-Populaire et al., 2000) and in 1999 in Taiwan (Yan et al., 2000). Unlike genetic determinants harboring *bla*<sub>CTX-M-3</sub> gene which were not sequenced at that time, CTX-M plasmid size and plasmid restriction patterns showed similarity demonstrating the potential diffusion of the same plasmids within isolates obtained from Poland and Taiwan (Yan et al., 2000). Despite this result, importation of this enzyme to Taiwan from Europe or vice versa was not possible to rule out as clonality was not addressed. Nevertheless, in both countries rapid dissemination of this enzyme was illustrated after its emergence (Baraniak et al., 2002; Yu et al., 2002; Ma et al., 2005).

Diversification of CTX-M-1 cluster was exemplified with increasing descriptions of new variants over the 1990s. For instance, CTX-M-10 was mainly described in the Mediterranean area (Spain and France; Oliver et al., 2001; Leavitt et al., 2009) and the CTX-M-15, initially identified in 1999 in enteric isolates recovered from a hospital in New Delhi, India (Karim et al., 2001), but nowadays found all over the world (Hawkey and Jones, 2009; Rogers et al., 2011). The CTX-M-10 differs from CTX-M-3 in two amino acids (A27V and R38Q) whereas CTX-M-15 in only one amino acid position (D240G). Nevertheless,

diversification of CTX-M-3, CTX-M-10, and CTX-M-15 should have been produced from a common ancestor (Novais et al., 2010).

A similar example is the CTX-M-9 enzyme, which was initially described in Spain and Brazil in 1996 (Sabaté et al., 2000; Bonnet et al., 2001), followed by China in 1997 (Chanawong et al., 2002). A retrospective study performed in France revealed the presence of this enzyme in strains isolated during 1994 (Saladin et al., 2002). In Spain, this enzyme became one of the most prevalent ESBL but co-existing with CTX-M-14 enzyme (a variant of CTX-M-9 differing in one amino acid, A231V). This enzyme emerged in Korea in 1995 (Pai et al., 2001) and was rapidly disseminated over the world. In China, it was detected in 1997–1998 (Chanawong et al., 2002), in Taiwan in 1998–1999 (Yu et al., 2002), in France in 1999 (Dutour et al., 2002) and in Brazil in 1999 (Bonnet, 2004).

Surveys performed in different countries during that period revealed that once a CTX-M  $\beta$ -lactamase penetrates in a specific geographic area it becomes prevalent with displacement or superimposition over TEM and SHV ESBL variants. This was the case of CTX-M-2 in South America, CTX-M-3 in Poland and CTX-M-14 in Spain, Taiwan and China (Bonnet, 2004; Livermore and Hawkey, 2005). This landscape was also the case for CTX-M-15 in United Kingdom (Paterson and Bonomo, 2005; Cantón and Coque, 2006; Hawkey and Jones, 2009). As previously noted, it was first isolated in India in 1999 (Karim et al., 2001) but also emerged in the United Kingdom in 2001 and probably in Japan early in the 2000s [designated as UOE-1 in GeneBank (accession number AY013478; Mushtaq et al., 2003; Livermore and Hawkey, 2005)]. Moreover, in Poland a retrospective study of a collection of Enterobacteriaceae resistant to expanded-spectrum cephalosporins revealed the presence of the CTX-M-15 in *Serratia marcescens* and *E. coli* since 1998 (Baraniak et al., 2002). Interestingly plasmid analysis suggested that this enzyme could have evolved directly from CTX-M-3 rather than being imported from another country and independently in different hospitals.

In the United Kingdom, isolation of CTX-M was rare before 2001 and only sporadic cases of CTX-M-9 were described as well as an important outbreak due to CTX-M-26, a variant of CTX-M-25 with three amino acid substitutions (V80A, Q225R, and G242D), which was previously described in Canada (Munday et al., 2004). The CTX-M-15 rapidly disseminated in the United Kingdom since its first detection. Isolates were particularly associated with different *E. coli* clones, A to F, the first one representing the majority and later identified as belonging to the international O25:H4-ST131 clone that has been detected all over the world (Lau et al., 2008). Again a dramatic increase of CTX-Ms was demonstrated mainly due to the CTX-M-15 enzymes (Coque et al., 2008b).

In the United States, CTX-Ms were rare during the 1990s but increased from 25% in 2000 to nearly 90% in 2005 (Lewis et al., 2007). Interestingly, CTX-M-15 rapidly penetrated over a minority of CTX-Ms that include CTX-M-8, CTX-M-14, and CTX-M-16 (Lewis et al., 2007; Johnson et al., 2010; Peirano et al., 2010). Within this country and as in other parts of the world, the CTX-M-15 enzyme has been identified in hospitalized and extrahospitalary patients and also in companion animals in which CTX-M-15 was the most prevalent CTX-M enzyme (Castanheira et al., 2008; Urban et al., 2010; Shaheen et al., 2011).

**Table 1 | Initial description of CTX-M enzymes belonging to different CTX-M groups.**

Year	Country	CTX-M	CTX-M group	Comment	Reference
1986	Japan	FEC-1	CTX-M-1	<i>Escherichia coli</i> from the fecal flora of laboratory dogs used for pharmacokinetic studies and previously administered cephem antibiotics	Matsumoto et al. (1988)
1989	Germany	CTX-M-1	CTX-M-1	<i>Escherichia coli</i> isolate recovered from the ear of a 4-month child suffering from otitis media in Munich. The $\beta$ -lactamases involved were firstly named as cefotaximases	Bauernfeind et al. (1990)
1989	Argentina	CTX-M-2	CTX-M-2	<i>Salmonella</i> Thypimurium isolates from hospitalized patients with meningitis, septicemia, or enteritis	Bauernfeind et al. (1992)
1989	France	MEN-1	CTX-M-1	<i>Escherichia coli</i> isolate in France in an Italian patient. Later sequencing reveals identity with CTX-M-1	Bernard et al. (1992), Barthélémy et al. (1992)
1993	Japan	Toho-1	CTX-M-2	<i>Escherichia coli</i> isolate recovered from a 1-year-old female child who developed cystitis. Toho-1 was later renamed as CTX-M-44	Ishii et al. (1995)
1996	Poland	CTX-M-3	CTX-M-1	<i>Citrobacter freundii</i> and <i>Escherichia coli</i> isolates recovered in Warsaw in patients with urinary tract infections	Gniadkowski et al. (1998)
1999	Taiwan	CTX-M-3	CTX-M-1	Different <i>Escherichia coli</i> isolates from several sources in hospitalized and non-hospitalized patients	Yan et al. (2000)
1994	France	CTX-M-9	CTX-M-9	A retrospective study showed presence of <i>Escherichia coli</i> with this enzyme in this country before its first recognition in Spain	Saladin et al. (2002)
1996	Spain	CTX-M-9	CTX-M-9	First description of CTX-M-14 in an <i>Escherichia coli</i> isolate from a patients with an urinary tract infection	Sabaté et al. (2000)
1996	Brazil	CTX-M-9	CTX-M-9	<i>Escherichia coli</i> isolate recovered from an hospitalized patient in Rio de Janeiro	Bonnet et al. (2001)
1997	China	CTX-M-9	CTX-M-9	<i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i> recovered as part of an antimicrobial resistance monitoring project	Chanawong et al., 2002
1996	Korea	CTX-M-14	CTX-M-9	Detected in <i>Shigella sonnei</i> and in blood isolates of <i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i>	Pai et al. (2001)
1997–1998	China	CTX-M-14	CTX-M-9	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> and <i>Enterobacter cloacae</i> recovered as part of an antimicrobial resistance monitoring project	Chanawong et al. (2002)
1998	Taiwan	CTX-M-14	CTX-M-9	<i>Escherichia coli</i> recovered during a multicenter antimicrobial resistance surveillance study	Ma et al. (2002)
1998	Poland	CTX-M-15	CTX-M-1	A retrospective study showed the presence of <i>Serratia marcescens</i> and <i>Escherichia coli</i> before the first description of this enzyme in India. Sequence and plasmid analysis revealed potential evolution from CTX-M-3	Baraniak et al. (2002)
1999	India	CTX-M-15	CTX-M-1	First description of this enzyme	Karim et al. (2001)
2001	United Kingdom	CTX-M-15	CTX-M-1	Spread in the community of <i>Escherichia coli</i> isolates with CTX-M-1. A major clone was later identified as belonging to the international 25:H4-ST131 clone	Mushtaq et al. (2003), Woodford et al. (2004)
1996–1997	Brazil	CTX-M-8	CTX-M-8	<i>Enterobacter cloacae</i> , <i>Citrobacter amalonaticus</i> , and <i>Enterobacter aerogenes</i> from different sources in hospitalized patients	Bonnet et al. (2000)
2000	Canada	CTX-M-25	CTX-M-25	<i>Escherichia coli</i> isolate recovered from an hospitalized patient	Munday et al. (2004)

The model of CTX-M-15 dissemination could have been different in countries in which other CTX-M  $\beta$ -lactamases were well established prior to the detection and penetration of this enzyme. This is the case of Spain and South America. In our institution in Spain, CTX-M-10 was first detected in 1990 in *K. pneumoniae*

and in 1991 in *E. coli*. The first detection of CTX-M-9 was in *E. coli* in 1996 and in *K. pneumoniae* in 1999, CTX-M-14 in 2001 in *K. pneumoniae* and in *E. coli* and CTX-M-15 in 2001 in *E. coli* and in 2002 in *K. pneumoniae*. It is of note that in a national survey in 2000 in Spain involving 40 centers, CTX-M-15 was not



found. However, in 2006 in a similar study, CTX-M-15 was found in different regions and was one of the most prevalent enzymes after CTX-M-14 and SHV-12 (Díaz et al., 2010). More recently, in our institution CTX-M-15 represents 34% of total ESBL producing *E. coli* (Novais et al., unpublished data), 37% of CTX-M-14, and 20% of SHV-12, denoting the increasing prevalence of CTX-M-15 but still co-existing with other CTX-M enzymes. This data illustrates that when CTX-M-15 penetrates in a landscape where other ESBLs are present the increment of this enzyme and displacement of other ESBLs is a slow process. This model can also be the case of South America where CTX-M-2 has been the most prevalent CTX-M over years. Nevertheless, reports of CTX-M-15 producing isolates are increasing in this continent, including Colombia (Valenzuela de Silva et al., 2006; Ruiz et al., 2011), Brazil (Cergole-Novella et al., 2010), Uruguay (Bado et al., 2010), and Peru (Pallecchi et al., 2004, 2007).

At present, we cannot rule out that international travel and immigration might have contributed during the 1990s and the early 2000s to the rapid emergence and dissemination of CTX-M enzymes in different countries and geographic areas. This has recently been demonstrated with carbapenemases and particularly with the NDM-1 metallo- $\beta$ -lactamase-producing organisms (Kumarasamy et al., 2010). Nevertheless, the presence of CTX-M enzymes in animals and most importantly in uncooked food products that were transported from one country to another suggests potential routes for dissemination (Matsumoto et al., 2007; Warren et al., 2008; Dhanji et al., 2010).

Finally, globalization of CTX-M enzymes is illustrated by their presence not only in food and companion animals but also in wild animals (Bonnedahl et al., 2010; Literak et al., 2010; Gonçalves et al., 2011; Silva et al., 2011). Additionally, they have been isolated in environmental compartments (Chen et al., 2010; Dhanji et al., 2011a).

### CTX-M ENZYMES NOT ONLY IN ENTEROBACTERIACEAE

As previously stated CTX-M enzymes were initially described in *E. coli*, *K. pneumoniae*, and *Salmonella* spp. but rapidly emerged in other Enterobacteriaceae. This event occurs in bacterial isolates with chromosomal mechanisms such as inducible chromosomal AmpC in *Enterobacter* spp., *S. marcescens*, *Citrobacter* spp., and *Morganella morganii* able to destroy oxy-imino-cephalosporins (Cantón et al., 2002; Paterson and Bonomo, 2005). This find suggests the facility for transmission of *bla*<sub>CTX-M</sub> genes and also that the acquisition of these genes could have lower fitness cost for the bacteria than selection of derepressed AmpC  $\beta$ -lactamase variants which finally also contribute to cephalosporin resistance.

As CTX-M enzymes increased in prevalence in Enterobacteriaceae in the early 2000s, they also increased in non-fermentative Gram-negative bacilli and other non-Enterobacteriaceae species. The first CTX-M enzyme described in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* was observed in isolates recovered in 2004 from sputum samples of a cystic fibrosis patient in Amsterdam (al Naiemi et al., 2006). The CTX-M enzyme from these isolates was characterized as CTX-M-1. In the same 2004 year, CTX-M-43 (a variant of CTX-M-44 carrying the D240G change and belonging to the CTX-M-2 group), was also observed in *P. aeruginosa* and *Acinetobacter* spp. in isolates recovered from

Bolivian hospitals (Celenza et al., 2006). CTX-M-2 was also found in a *P. aeruginosa* isolate recovered in a patient hospitalized in Brazil in 2005 (Picão et al., 2009). Despite these descriptions, prevalence of CTX-M enzymes in non-fermenter bacilli is still rare. This fact could be due to incompatibility of plasmids carrying these enzymes or might reflect phenotypic difficulties in recognizing non-fermenter isolates expressing CTX-Ms. On the other hand, *Aeromonas* spp. or *Vibrio* spp. isolates with CTX-M enzymes have been also recognized (Soler Bistué et al., 2006; Ye et al., 2010; Chowdhury et al., 2011; Gómez-Garcés et al., 2011).

### ORIGIN AND MOBILIZATION OF THE *bla*<sub>CTX-M</sub> GENES

According to phylogenetic trees performed with all CTX-M  $\beta$ -lactamases described, the CTX-M lineage could be differentiated at in least five main clusters (Figure 3). Traditionally, each CTX-M cluster has been related to the chromosomal *bla* genes present in different *Kluyvera* species, which are part of the human normal intestinal microbiome, usually at low bacterial counts, and is considered as a saprophytic and an opportunistic pathogen (Farmer et al., 1981). Furthermore, they have been occasionally isolated in humans associated with different infections, mainly affecting the urinary tract and skin and soft tissues (Sarria et al., 2001) and is also present in the environment as free-living organisms in water, soil, sewage, and food products of animal origin (Farmer et al., 1981).

Chromosomal *bla* gene, *kluC*, present in *Kluyvera cryocrescens* has been considered the ancestor of the CTX-M-1 cluster (Decousser et al., 2001), *kluA* gene from *Kluyvera ascorbata* the origin of the CTX-M-2 cluster (Humeniuk et al., 2002) and three different chromosomal *bla* genes from *Kluyvera georgiana*, *kluG*, *kluY*, and *bla*<sub>CTX-M-78</sub> the origin of CTX-M-8 (Poirel et al., 2002), CTX-M-9 (Olson et al., 2005), and CTX-M-25 clusters (Rodríguez et al., 2010) respectively. Interestingly, the CTX-M-74 and CTX-M-75, characterized in a survey conducted in Brazil and with single one amino acid change with respect to CTX-M-2 (Minarini et al., 2009), showed a degree of nucleotide divergence that could be considered as a new cluster, reflecting a convergent evolution of these  $\beta$ -lactamases (Stepanova et al., 2008). All these data suggest that the environmental source of CTX-M  $\beta$ -lactamases was the genus *Kluyvera*. Although the scientific community has unequivocally accepted this point, several questions still arise to guarantee this line of evidence. First, the comparison of the topologies of phylogenetic trees between *bla*<sub>CTX-M</sub> and available 16S rDNA sequences of *Kluyvera* species reveals that the evolutionary relationships among CTX-M clusters is not completely correlate with the phylogenetic distance among the different species of *Kluyvera* (Figure 3). Whereas all *Kluyvera* species diversified at the same time from the same ancestral bacteria, this topology is not observed among *bla*<sub>CTX-M</sub> clusters, suggesting different evolutionary trajectories. Second, if each chromosomal gene from *Kluyvera* was the source of a determined CTX-M cluster, why have at least three chromosomal genes from *K. georgiana* (*bla*<sub>kluG</sub>, *bla*<sub>kluY</sub>, and *bla*<sub>CTX-M-78</sub>) been proposed as precursor of three different CTX-M clusters? On the contrary, CTX-M-1 cluster has been related to different *Kluyvera* species (*K. ascorbata* and *K. cryocrescens*; Decousser et al., 2001; Rodríguez et al., 2004).



April 2012 | Volume 3 | Article 110 | 7

**FIGURE 3 | Continued**

**Maximum likelihood trees of *bla*<sub>CTX-M</sub> genes and 16S rDNA of *Kluyvera* spp. in order to compare their respective phylogenetic topologies. (A)** Phylogenetic tree of *bla*<sub>CTX-M</sub> genes ( $n = 109$  and 17 chromosomal genes from *Kluyvera*) was obtained using PhyML\_3.0 program (846 nt). The Tamura Nei nucleotide substitution model, used as evolutionary model was selected with the jModeltest program. The robustness of the relevant nodes was estimated with 1000 bootstrap

pseudorandom replicates. We considered nodes valid when bootstrap value was  $>95\%$ . **(B)** Phylogenetic tree of 16S rDNA of *Kluyvera* spp. ( $n = 55$ ) and related species download of www.ncbi.nlm.nih.gov, using PhyML\_3.0 (1310 nt). The Hasegawa-Kishino-Yano was the evolutionary model inferred by jModeltest program and invariant site rate of  $77.6\%$  (HKY + I + G). We considered nodes valid when bootstrap value was  $>85\%$ . The phylogenetic trees were represented using MEGA 5.0 program.

These data could also suggest that *bla*<sub>CTX-M</sub> genes have been also circulating among different *Kluyvera* species. Third, upstream of *bla*<sub>klu</sub> gene identical genome organization was found in *Enterobacter aerogenes*, suggesting that *Kluyvera* could have gained *bla*<sub>klu</sub> gene after these species diverged, therefore most of the isolates must carry *bla*<sub>klu</sub> gene, including *Kluyvera intermedia*, where no *bla*<sub>klu</sub> gene has been identified. However, despite the presence of the *bla*<sub>klu</sub> gene in most *Kluyvera* isolates, 90% are susceptible to cefotaxime (Sarría et al., 2001). Obviously, *bla*<sub>klu</sub> gene could be present but weakly expressed. Unfortunately the presence of *bla*<sub>klu</sub> gene in *Kluyvera* susceptible isolates or their genetic environment has not been searched. These studies could help us to understand previous observations of *Kluyvera* spp. as the precursor of the CTX-M enzymes and responsible for the current pandemic.

It is well known that chromosomal *bla*<sub>klu</sub> genes are weakly expressed in their *original* context and require the presence of a strong promoter upstream to increase the MIC values and consequently be considered as phenotypically resistant. The insertion sequences (IS) have provided this promoter in clinical cefotaxime-resistant *Enterobacteriaceae* strains, the most prevalent being *ISEcp1* and *ISCR1*, while *IS26* has been only detected in few isolates. Curiously, these IS have not been described in natural isolates of *Kluyvera*, although the capture of *ISEcp1* upstream of chromosomal *bla*<sub>klu</sub>/CTX-M genes occurs at high frequency in laboratory conditions. This was demonstrated in an elegant *in vitro* experiment performed by Lartigue et al. (2006). When *ISEcp1* was inserted upstream of *bla*<sub>klu</sub>/CTX-M gene, stress situations such as high temperature ( $40^{\circ}\text{C}$ ) or that exerted by antimicrobials such as ceftazidime or cefotaxime, the mobilization of *ISEcp1* was increased, carrying the downstream sequences. It has been speculated that global warming or higher temperature than humans in the intestinal compartment of certain animals and the increasing use of antimicrobials might have also contributed to the extraordinary increase and spread of the *bla*<sub>klu</sub>/CTX-M genes (Lartigue et al., 2006; Nordmann et al., 2008). Apart from these speculations, an interesting question is if mobilization of *bla*<sub>klu</sub> occurred, how often was it in the nature? Initially and accepting the genus *Kluyvera* as progenitor of the CTX-M enzymes, we can suspect mobilization of the same number of occasions has CTX-M clusters has been described. Barlow et al. (2008) using phylogenetic trees based on gene distance suggest that this occurred at least eight times (three times each for CTX-M-1 and CTX-M-2 clusters and only one each for CTX-M-9 and CTX-M-8; Barlow et al., 2008). However the description of different IS such as *ISEcp1* and *ISCR1* related to CTX-M-9 (Valverde et al., 2009), allow us to suspect that more mobilization events may have occurred (see below).

## EVOLUTION AND DIVERSIFICATION OF CTX-M $\beta$ -LACTAMASES

Cumulative representation of the new variants described among the most prevalent  $\beta$ -lactamases shows that the highest slope during the last years correspond to the CTX-M and OXA-type  $\beta$ -lactamases (Bush and Fisher, 2011). This might indicate that they have recently suffered the most spectacular diversification process in the  $\beta$ -lactamases world. CTX-M phylogenetic trees suggest an explosive diversification from the original *bla* genes in a short time when they arrived in the clinical scenario (Novais et al., 2010). The first CTX-Ms identified were able to confer cefotaxime resistance although their capacities to efficiently hydrolyze ceftazidime was very low or null, but nowadays more than 60% of CTX-M variants are able to confer cefotaxime and ceftazidime-resistance simultaneously. Thus, ceftazidime could have been one of the main selective forces contributing to the diversification of CTX-Ms.

An evolution experiment was designed in our laboratory to obtain more efficient CTX-M variants against ceftazidime; the ceftazidime-resistant CTX-M variants carried the changes A77V, P167S, and D240G (Novais et al., 2008). These changes have been described in the nature in several cases including CTX-M-52 and CTX-M-87, which derived from CTX-M-15 and CTX-M-14 respectively (Yin et al., 2009). These results reinforce the idea that ceftazidime has driven the selection of new CTX-M variants. Moreover, we calculated the ratio of the number of non-synonymous substitutions with respect to synonymous substitutions ( $\omega = dN/dS$ ) among the *bla*<sub>CTX-M</sub> gene variants in order to define positive (accelerate evolution) or purified (slow evolutionary rate) selection processes during the diversification of CTX-Ms. In the deep branches of the CTX-Ms phylogenetic tree, processes of purified selection occurred while positive selection was the mechanism of molecular evolution in the more recent branches. In our experiments, six positions were identified to be under positive selection, including the amino acid positions 77, 167, and 240, which were affected under ceftazidime regimens. Nevertheless, our results also suggest the presence of more evolutionary forces involved in the selection of new and better adapted variants. The construction of mutants carrying combinations of these mutations revealed that the simultaneous presence of cefotaxime and ceftazidime in the environment has modeled the explosive evolution and diversification of the CTX-M enzyme (Novais et al., 2010).

Recombination events also accelerate the evolution. These events have also been described among *bla*<sub>CTX-M</sub> genes. As an example, the CTX-M-64 enzyme is the result of a recombination between members of the *bla*<sub>CTX-M-9</sub> and *bla*<sub>CTX-M-1</sub> derived genes, probably *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub>, as they are the most prevalent variants in the world (Nagano et al., 2009). The recombination



requires the simultaneous presence of both *bla*<sub>CTX-Ms</sub> in a same habitat. Recently, we described in an Ethiopian 1-year-old boy both CTX-M-14 and CTX-M-15 (Morosini et al., 2010). Moreover, in a national surveillance performed in China, the CTX-M-64  $\beta$ -lactamase was recovered from three patients co-existing with other CTX-Ms (Sun et al., 2010). As the concomitant presence of different CTX-M  $\beta$ -lactamases in the same bacteria is no longer an unusual event, other recombinant enzymes might emerge in the near future.

### ***bla*<sub>CTX-M</sub> GENE PLASTICITY AND OTHER STRATEGIES TO INCREASE THE SPECTRUM OF ACTION OF CTX-M $\beta$ -LACTAMASES**

$\beta$ -Lactamases are highly modeling proteins as few positions are critical to hydrolyze  $\beta$ -lactam compounds. Nowadays, at least 136 polymorphic positions have been found among all CTX-Ms described. Some of these changes play a main role increasing the  $\beta$ -lactam hydrolytic spectrum while others modulate or restore the synergic or antagonist effects caused by other modifications. Furthermore, some mutations are selected depending on the genetic background due to the interactions among the mutations present (epistasis) whereas other changes have an unknown effect on the enzyme and they could be only selected by random drift. For instance, directed mutagenesis studies have revealed new mutations that have not yet been described in nature, which could be selected under ceftazidime challenge such as M135I and A219V (Pérez-Llarena et al., 2011). These results suggest that the adaptive possibilities of CTX-M enzymes have not yet reached their evolutionary stasis.

In this section we explore if other mutations or adaptive possibilities could be expected in the CTX-M lineage. Two attractive issues are raised in relation to the capacities of CTX-Ms to confer resistance to non-oxyimino- $\beta$ -lactam antibiotics, including  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations (IR-CTX-M) and/or carbapenems (Girlich et al., 2008; Ripoll et al., 2011), as these variants have not yet been found in nature. In laboratory conditions, IR-CTX-M mutants carrying the S130G, K234R, and S237A mutations have been described (Gazouli et al., 1998; Aumeran et al., 2003; Ripoll et al., 2011). The selection of these mutants conferring resistance to  $\beta$ -lactam plus  $\beta$ -lactamase inhibitors showed an antagonistic pleiotropic effect with a simultaneous loss of the hydrolytic activity against cefotaxime and ceftazidime. Nevertheless, the L169S mutation that was recovered after the S130G was selected, slightly increase the hydrolytic efficiency against cefotaxime and ceftazidime, suggesting that mutations at position 169 could have a restoring effect, similar to the selection of A77V mutation after the emergence of P167S or D240G driving ceftazidime-resistance (Novais et al., 2008; Ripoll et al., 2011). Interestingly, a clinical isolate carrying a novel CTX-M variant (CTX-M-93), which differs from CTX-M-27 in a single L169Q mutation, shows a reduced activity against cefotaxime but increased activity against ceftazidime when compared with its progenitor (Djamdjian et al., 2011). This finding confirms that the mutations in 169-position confers increased hydrolytic activity against ceftazidime and this improvement is higher when the S130G mutation is also present. On the other hand, a recent study using a site-directed mutagenesis strategy found that the V103D and V260L mutations also

endow an IR-CTX-M phenotype (Pérez-Llarena et al., 2011), again suggesting that new mutants could be selected in the future.

No mutations in *bla*<sub>CTX-M</sub> gene have been involved in resistance to carbapenems. However, clinical carbapenem-resistant strains carrying CTX-M  $\beta$ -lactamases have been isolated. Two types of mutants are related to this carbapenem-resistant phenotype: loss of porins and co-existence with other  $\beta$ -lactamases (Gülmez et al., 2008). The loss of OmpK35 and OmpK36 porins is the most frequent mechanism of carbapenem-resistant associated with CTX-M producing *K. pneumoniae* (Leavitt et al., 2009) and its functional restoration significantly decreased the MICs of all carbapenems (Doumith et al., 2009). *In vivo* resistance development to carbapenem has been previously described in two *K. pneumoniae* strains producing CTX-M-1 due to the lack of OmpK36 expression (Mena et al., 2006). However, the loss of this porin reduced *in vitro* fitness and attenuated virulence in a murine model (García-Sureda et al., 2011). Moreover, detection of carbapenem-resistant *E. coli* clinical strains carrying CTX-M due to reduced expression of OmpF and OmpC porins, homologous of OmpK35 and OmpF36, are increasingly recognized (Girlich et al., 2009; Bennett et al., 2010). These results illustrated that porin deficiency is more efficient than potential CTX-M mutation for the increase of carbapenem MICs.

On the other hand, the high worldwide distribution of CTX-M enzymes is giving rise to the co-existence of two or more  $\beta$ -lactamases in the same strain. This possibility is becoming a common bacterial strategy to enhance antibiotic resistance. CTX-Ms have been described associated with VIM-1 (Miró et al., 2010), KPC-2 (Chen et al., 2011), or OXA-48 carbapenemases (Cuzon et al., 2011; Pitart et al., 2011). In fact, the clone belonging to ST395 coproducing CTX-M-15 and OXA-48 has been demonstrated widespread throughout Europe (Potron et al., 2011). An extreme description was the co-existence of KPC-2, VIM-19, CMY-2, and CTX-M-15 in a *K. pneumoniae* strain isolated in Greece (Pournaras et al., 2010). Moreover, the recently described NDM-1 carbapenemase is normally present in isolates also expressing the CTX-M-15 ESBL and also OXA-type enzymes (Poirel et al., 2011a; Solé et al., 2011). This supports the concept of the genetic capitalism of the multi-drug resistant bacteria where the more resistant bacteria have the higher opportunity to become more resistant (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

### **GENETIC ENVIRONMENTS OF *bla*<sub>CTX-M</sub> GENES PARTICIPATING IN THE MOBILIZATION, SPREAD, AND MAINTENANCE OF THESE GENES**

A linkage of different *bla*<sub>CTX-M</sub> genes with specific surrounding genetic elements, including IS, integrons, and transposons has been described. These genetic backbones are integrated in more complex structures such as different plasmid replicon types as well as specific clones defining a complex hierarchical organization with the possibility of interchanging different modules (Cantón and Coque, 2006; Carattoli, 2011; Woodford et al., 2011). **Figure 2** shows hierarchical complexity of *bla*<sub>CTX-M</sub> genes within different genetic structures and bacterial clones participating in the mobilization, spread, and maintenance of these genes. As previously described, *bla*<sub>CTX-M</sub> genes have been hypothetically mobilized

from *Kluyvera* spp. by IS and to a lesser extent by bacteriophages. The IS also participate in the over-expression of these genes and some of them (i.e., *ISCR1*) are adjacent to integron structures that are also integrated in transposition units. These supra-structures are often incorporated within conjugative plasmids that might be present in successful clones (or the so-called high-risk clones). Moreover, they can operate as evolutionary units and might individually act as units of selection (Baquero, 2004; Cantón and Ruiz-Garbajosa, 2011).

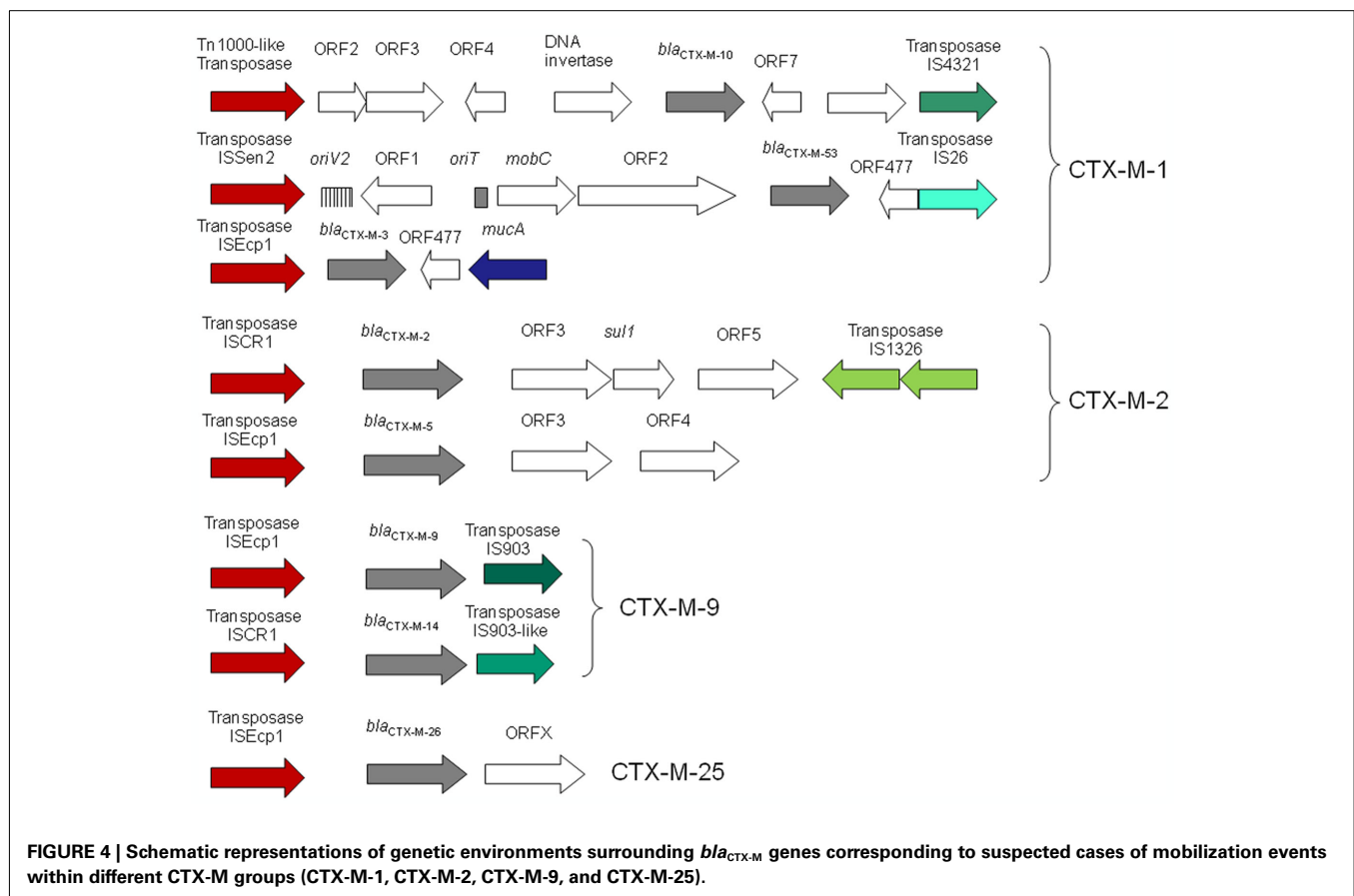
Some of the genetic elements and plasmids harboring *bla*<sub>CTX-M</sub> genes also harbor other resistance genes, including those encoding AmpC  $\beta$ -lactamases (plasmid *bla*<sub>AmpC</sub>) and carbapenemases, plasmid-mediated quinolone resistance (PMQR) genes (i.e., *qnr* genes), or methylases affecting aminoglycosides. All these genes might also give advantage to *bla*<sub>CTX-M</sub> for maintenance due to co-selection processes.

### INSERTION SEQUENCES AND PHAGE-RELATED SEQUENCES INVOLVED IN THE MOBILIZATION OF *bla*<sub>CTX-M</sub> GENES

There are a huge number of scientific articles describing *new* or *unique* genetic rearrangement associated with *bla*<sub>CTX-M</sub> genes but further analysis has revealed that they are only variations of a few different genetic structures (Lartigue et al., 2004; Eckert et al., 2006; Dhanji et al., 2011b; Toleman and Walsh, 2011). The experimental mobilization of *bla*<sub>CTX-Ms</sub> genes have been demonstrated by IS located upstream such as *ISEcp1* (Lartigue et al., 2006). Moreover, the effect of *P*<sub>out</sub> promoter of these IS increasing the *bla*<sub>CTX-Ms</sub>

expression suggests that the IS located upstream of these genes have a role in the selection and dissemination of CTX-Ms. Therefore, we have mainly considered in this part of the review the genetic upstream sequences of the *bla*<sub>CTX-M</sub> genes. The information of downstream sequences of *bla*<sub>CTX-M</sub> genes is less available and less knowledge has been accumulated.

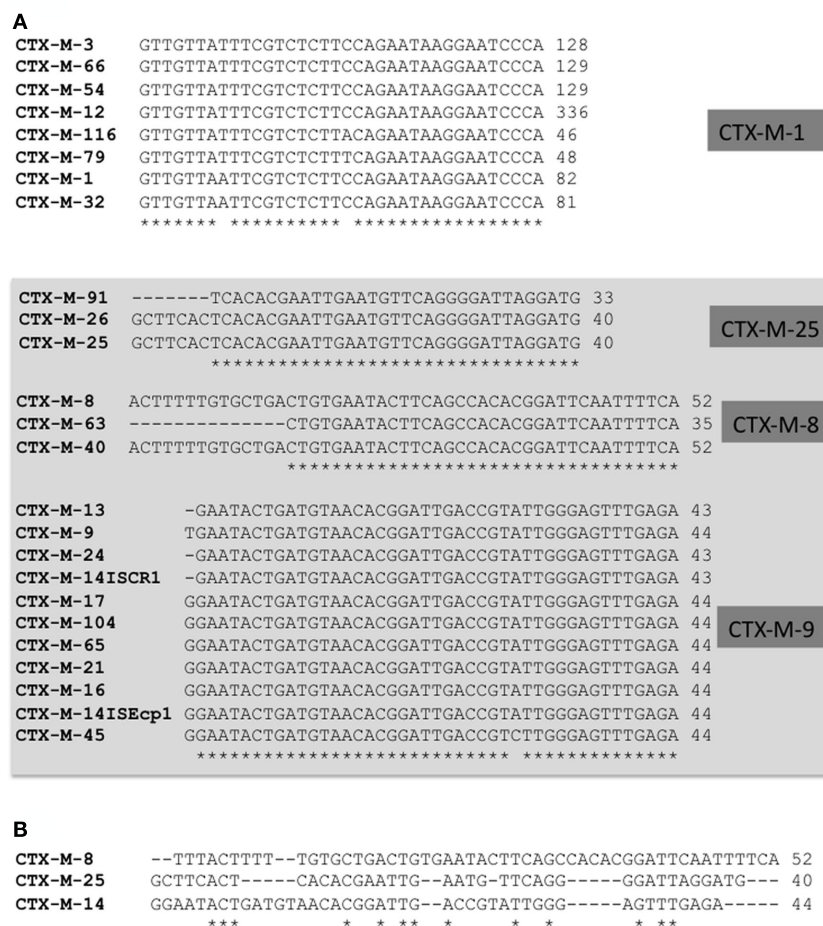
Different IS have been identified upstream of the *bla*<sub>CTX-Ms</sub> genes, including *ISEcp1*, *ISCR1*, *IS10*, and *IS26*. Although other IS elements have also been described upstream, they were the result of subsequent integration events. For instance, *IS1* and *IS10*, and even *ISCR1* and *IS26*, have been described interrupting *ISEcp1* (Eckert et al., 2006; Bae et al., 2008). *ISEcp1* is the most widely found IS upstream of different *bla*<sub>CTX-M</sub>. Although it was first recorded adjacent to the *bla*<sub>CTX-M-15</sub> in 1999, it has been found associated with all clusters of these  $\beta$ -lactamases except CTX-M-8. Moreover, *ISCR1* has been related to several members of *bla*<sub>CTX-M-2</sub> and *bla*<sub>CTX-M-9</sub> clusters and *IS10* with *bla*<sub>CTX-M-8</sub>. The number of new genetic environments upstream of *bla*<sub>CTX-M</sub> must be related to the number of mobilization events but unfortunately in many *bla*<sub>CTX-M</sub> cases the upstream sequence is unknown (Barlow et al., 2008). Nowadays, we have been able to identify at least nine mobilization events (Figure 4): three in CTX-M-1 cluster (*bla*<sub>CTX-M-10</sub>, *bla*<sub>CTX-M-53</sub>, and other known *bla*<sub>CTX-M</sub> genes included in this cluster), two in CTX-M-2 cluster (*bla*<sub>CTX-M-2</sub> and *bla*<sub>CTX-M-5</sub>), two in CTX-M-9 cluster (*bla*<sub>CTX-M-9</sub> and *bla*<sub>CTX-M-14</sub>), and one each in CTX-M-8 and CTX-M-25 (*bla*<sub>CTX-M-8</sub> and *bla*<sub>CTX-M-25</sub>, respectively).



On the other hand, spacer sequences between *ISEcp1* and *bla*<sub>CTX-M</sub> genes have been well studied. The genetic distance between IS and *bla*<sub>CTX-M</sub> gene is related to cephalosporin MIC values (Ma et al., 2011). These distances ranged between 48 and 127 bp in *bla*<sub>CTX-M-1</sub> gene cluster, between 34 and 42 bp in *bla*<sub>CTX-M-9</sub> gene cluster (but more than 300 bp if *ISCR1* is upstream of *bla*<sub>CTX-M</sub>) and around 40–52 in *bla*<sub>CTX-M-25</sub> and *bla*<sub>CTX-M-8</sub> gene clusters respectively. When the homology among spacer sequences of *bla*<sub>CTX-M-1</sub> gene cluster was analyzed, all but *bla*<sub>CTX-M-10</sub> and *bla*<sub>CTX-M-53</sub> maintained a common region (known as V and W sequences). This suggests that these *bla*<sub>CTX-M</sub> genes belonging to CTX-M-1 cluster could derive from a single transposition event. A similar conclusion can be obtained when considering *ISEcp1* and *bla*<sub>CTX-M</sub> genes belonging to *bla*<sub>CTX-M-9</sub> (Y sequence), *bla*<sub>CTX-M-8</sub> and *bla*<sub>CTX-M-25</sub> (Figure 5). In all these clusters, the ancestral source was *K. georgiana*. However the lack of homology among spacer sequences of these *bla*<sub>CTX-M</sub> gene clusters suggests a different source in each case. These results reveal that non-described *Kluyvera* species (or another organism source) could be the origin of clusters ascribed to *K. georgiana* (see Figure 3

about the phylogenetic tree of 16S rDNA of *Kluyvera* spp. where more species of *Kluyvera* can be suggested according to bootstrap values).

Finally, *bla*<sub>CTX-M-10</sub> gene has been associated with phage-related sequences, which underlines bacteriophages involvement in the potential mobilization of this gene (Oliver et al., 2001). Although the occurrence of this event has not been frequently demonstrated, the importance of bacteriophages in the diffusion of *bla* genes was evaluated in sewage, showing that these sequences may contribute to the spread of some  $\beta$ -lactamases (Muniesa et al., 2004; Colomer-Lluch et al., 2011). Part of these sequences, also known as “phage-like elements,” could have facilitated mobilization of resistance genes into the chromosome (Wozniak and Waldor, 2010; Guglielmini et al., 2011). With the exception of the previous description of the CTX-M-10 enzyme (Oliver et al., 2001), these structures have not been associated with other CTX-Ms despite the fact that these enzymes have also been observed integrated into the chromosome (Coelho et al., 2010; Song et al., 2011). Future *in silico* analysis might reveal a more frequent presence of these phage-like elements associated with *bla* genes.



**FIGURE 5 | Alignment of upstream sequences in different *bla*<sub>CTX-M</sub> gene clusters.** Different *bla*<sub>CTX-M</sub> genes belonging to the same cluster share an identical DNA sequence in upstream region, suggesting the same origin (A). Nevertheless, the loss of alignment among different upstream

sequences from different *bla*<sub>CTX-M</sub> genes belonging to different clusters (included in the box squared) but theoretically derived from the same ancestral source in *Kluyvera georgiana* suggesting that these clusters had different sources (B).

## OTHER INTEGRATIVE AND MOBILIZATION UNITS

Recently, there has been an increasing interest for the so-called “integrative and conjugative elements” (ICEs) in the mobilization of resistant determinants. They are not homogenous structures and include the “conjugative transposons” or the so-called “integrated conjugative plasmids.” These elements are a diverse group of mobile genetic elements found in both Gram-positive and Gram-negative bacteria. They primarily reside in the chromosome, but retain the ability to excise from and re-integrate into a new chromosome like bacteriophages and to transfer between bacterial cells by conjugation like plasmids. It has been recently stressed that they are closely linked to some of the more powerful resistance mechanisms such as the extended-spectrum-, metallo-, and AmpC type  $\beta$ -lactamases (Toleman and Walsh, 2011).

A characteristic of the ICE elements, as well as complex class 1 integrons and some IS is the inability to consistently recognize one of their own terminal sequences, while they recognize more genetically distant surrogate sequences. This has the effect of mobilizing the DNA sequence found adjacent to their initial location. This process is known as one-ended transposition mechanisms and is exemplified by some IS such as *ISCR1* and *ISEcp1* as well as *Tn3* and *Tn21* derivatives, including *Tn5090*-like transposon (also called *Tn402*) that in turn harbor the class 1 integrons, *ISCR1* and certain *bla*<sub>CTX-M</sub> genes. A detailed review of the importance of these elements in the emergence and dispersion of resistance traits, including that exerted by *bla*<sub>CTX-M</sub> genes has been recently reviewed (Toleman and Walsh, 2011).

A novel structure named IMU that resides in a plasmid 7-kb IncQ-type plasmid has recently been associated with mobilization of *bla* genes (Poirel et al., 2009). The IMU structure resembles that of miniature inverted transposable elements (MITEs), which have been identified in eukaryotes such as plants (Chen et al., 2008) and also in bacteria (Buisine et al., 2002; Filée et al., 2007). In Enterobacteriaceae, they form a composite like element consisting of 288-bp elements located in opposite orientation able to mobilize defective class 1 integron structures carrying *bla* genes. Although they have not yet been associated with mobilization of *bla*<sub>CTX-M</sub> genes, they might participate in the future in the mobilization of these genes.

## PLASMIDS AND SPREAD OF *bla*<sub>CTX-M</sub> GENES

The importance of plasmids in the spread of *bla*<sub>CTX-M</sub> genes is exemplified with certain CTX-M  $\beta$ -lactamases and has been stressed in several publications (Carattoli, 2009). The case of CTX-M-15 enzyme is of particular interest. It has been shown that the *bla*<sub>CTX-M-15</sub> gene is mainly associated with incompatibility group FII plasmids (Coque et al., 2008b). They are a paradigm of narrow host-range plasmids characterized by a low-copy number in the bacteria. They are mainly found in Enterobacteriaceae and have recently been termed as “epidemic resistance plasmids” due to their propensity to acquire resistance genes and transfer among bacteria (Carattoli, 2011). The FII plasmids are not a homogeneous group and might have evolved by recombinatorial events among different plasmids. It has been shown that within these plasmids different replicon types are present that might facilitate fast evolution and plasmid diversification. Interestingly, initial studies on IncFII plasmids suggest that they were widely distributed within

Enterobacteriaceae before antimicrobial use provided that they were well adapted to these organisms (Datta et al., 1980). This fact has undoubtedly facilitated persistence and spread of resistant determinants, including *bla*<sub>CTX-M</sub> genes after this acquisition. Moreover, incorporation of genetic determinants mediating non  $\beta$ -lactam resistance mechanisms would have also contributed to the maintenance of these plasmids within the bacteria and hence the *bla*<sub>CTX-M</sub> genes under co-selection process (Novais et al., 2007; Woodford et al., 2009).

On the other hand, broad host-range replicon plasmids such as IncN, IncI1, and IncL/M have also been involved in the dissemination and spread of CTX-M enzymes. Some of these plasmids, such as IncN, might have their reservoir in animals as they have been isolated in *Salmonella* spp. and *E. coli* from food producing animals (Carattoli, 2011). They often carry *qnr* and *aac(6)-Ib-cr* genes determining low level fluoroquinolone resistance (Shen et al., 2008; Bado et al., 2010). More recently, these plasmids have been able to acquire genes coding the emerging carbapenemase such as *bla*<sub>NDM-1</sub> (Poirel et al., 2011b) and have been identified as epidemic plasmids in countries with a high prevalence of carbapenem resistance (Psichogiou et al., 2008). Other plasmids, such as IncL/M group are also able to carry *bla*<sub>CTX-M</sub> genes (i.e., *bla*<sub>CTX-M-3</sub> and *bla*<sub>CTX-M-15</sub>) as well as those producing methylase encoding genes involved in aminoglycoside resistance (i.e., *armA*; Sabtcheva et al., 2008; Naas et al., 2011).

Other plasmid types that have been preferentially associated with *bla*<sub>CTX-M</sub> genes are IncK plasmids with *bla*<sub>CTX-M-14</sub> in southern Europe (Valverde et al., 2009; Cottell et al., 2011) and IncHI2 plasmids with *bla*<sub>CTX-M-2</sub> and *bla*<sub>CTX-M-9</sub> in other geographic areas (García-Fernández et al., 2007). Nevertheless, these plasmids have also been responsible for the dispersion of other resistance genes such as IncK and *bla*<sub>CMY</sub> genes (Baudry et al., 2009) or IncHI2 and *bla*<sub>VIM</sub> and *qnr* genes (García-Fernández and Carattoli, 2010; Oteo et al., 2010b). These results denote ability of these plasmids for recruitment resistance genes, hence facilitating the increasing prevalence and persistence of the resistance traits of the bacteria. Furthermore, it is expected that persistence over time also gives opportunity to the bacteria to generate gene variation and an increasing number of *bla*<sub>CTX-M</sub> variants in the coming years as has occurred with OXA-type  $\beta$ -lactamases. Some of these variants have been obtained *in vitro* under antibiotic selective pressure but have not yet been found in clinical isolates (Novais et al., 2008, 2010; Ripoll et al., 2011).

## CLONAL DISPERSION AND CO-SELECTION PROCESSES: INVOLVEMENT OF MULTI-DRUG RESISTANT AND VIRULENT HIGH-RISK CLONES

One of the most interesting issues in the dispersion of CTX-M enzymes is the participation of specific clones, particularly those from *E. coli* and *K. pneumoniae*. Recent approaches based on MLST typing have demonstrated that despite a high diversity among CTX-M producers, a few clones or sequence types (ST) grouped in clonal complexes (CC) have been repeatedly found linked to CTX-M enzymes. This suggests that they are involved in the dissemination of these enzymes and that the adaptive success of some CTX-M enzymes could also depend on specific ST or CC where they are frequently present. These clones have been



named as high-risk clones (Woodford et al., 2011). The case of worldwide CTX-M-15 producing *E. coli* isolates exemplifies this issue as most of these isolates belong to the internationally spread ST131 clone (Coque et al., 2008b; Woodford et al., 2011). ST131 is the predicted founder of a clonal cluster that comprises 13 single locus variants (SLVs) and three double-locus variants (DLVs). It is related to serotype O25:H4 as well as to phylogenetic group B2, which grouped virulent extraintestinal isolates (Coque et al., 2008b; Peirano and Pitout, 2010). In addition, isolates belonging to this clone or CC frequently carry IncFII plasmids suggesting a common co-evolutionary process.

Different population structure studies of *E. coli* demonstrated that ST131 clone is also not only associated with CTX-M enzymes belonging to group 1 (CTX-M-1, CTX-M-3, and CTX-M-10) but also with others from distinct groups, such as CTX-M-9 or CTX-M-14 (Mora et al., 2010; Cao et al., 2011). This spread is present among human and animal isolates. In addition, very recent studies also showed that ST131 is over represented within non-ESBL producing isolates (Blanco et al., 2011) and that this clone can recruit emerging  $\beta$ -lactamases such as NDM-1 (Peirano et al., 2011). Multiresistance might have facilitated globalization of this clone. Moreover, at individual level the presence of specific virulence traits facilitating host adherence and receptor epithelial binding also contribute to persistence (Peirano and Pitout, 2010).

Other lineages of *E. coli* that have also contributed to the spread of CTX-M enzymes are ST38, ST393, and ST405 clones and their corresponding single- and DLVs (Coque et al., 2008a; Blanco et al., 2011). These lineages belong to serotype D, which represents also virulent extraintestinal strains (Picard et al., 1999). ST38 and ST393 seem to have been more preferential for the CTX-M-9 and CTX-M-14 enzymes whereas ST405 have been more preferentially with CTX-15 enzyme. Again these clones might also be overrepresented within non-ESBL producing isolates increasing the opportunity to recruit the corresponding *bla*<sub>CTX-M</sub> genes. These clones also express other emerging  $\beta$ -lactamases such as OXA-48 and NDM-1 as illustrated with ST38 (Poirel et al., 2011a; Yamamoto et al., 2011).

Many isolates belonging to these *E. coli* clones are fluoroquinolone-resistant uropathogenic isolates that have their natural environment in the intestinal microbiota. Fluoroquinolones might favor co-selection phenomena that could enrich the intestinal microbiota with these isolates. It has even been speculated that fluoroquinolone resistance either due to plasmid-mediated genes, including *qnr* variants and *acc(6′)-Ib-cr*, or to topoisomerase mutations preceded the acquisition of *bla*<sub>CTX-M</sub> genes (Jones et al., 2008; Naseer and Sundsfjord, 2011). In addition to this resistant trait, high-risk clones expressing CTX-M enzymes are also more favorable for acquiring other resistance mechanisms, such as those affecting aminoglycosides, trimethoprim-sulfamethoxazole and fosfomycin. Aminoglycoside resistance is related both with aminoglycoside modifying enzymes and methylases (Mushtaq et al., 2011). Fosfomycin resistance involving different resistance mechanisms has been more recently describe in isolates from the community in countries with increasing use of this antibiotic (Oteo et al., 2009). This again illustrates the genetic capitalism of the resistant bacterias (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

Together with these points, these CTX-M associated *E. coli* clones are highly virulent, easily transmitted among family members in colonized or infected patients (Mihaila et al., 2010) and have been found in wild-life, companion and food animals (Mora et al., 2010; Platell et al., 2011).

In *K. pneumoniae* more attention has been paid to carbapenemases, mainly KPCs, and the involvement of ST258 clone in their dissemination (Woodford et al., 2011). Nevertheless, different publications have studied the implication of different clones in the dissemination of CTX-M enzymes associated with *K. pneumoniae*. This is for instance the case of CC11, which has been identified widely spread in Asia associated with different CTX-M enzymes, including CTX-M-14 and CTX-M-15 (Ko et al., 2010; Lee et al., 2011). In Hungary this CC and also ST15 and ST147 were found to be responsible for the country-wide dissemination of ciprofloxacin-resistant CTX-M-15 producing *K. pneumoniae* (Damjanova et al., 2008). Also in the Copenhagen area of Denmark a *K. pneumoniae* ST16 clone harboring the *bla*<sub>CTX-M-15</sub> and *bla*<sub>SHV-28</sub> genes was recognized as producing hospital outbreaks. (Nielsen et al., 2011). Some of these clones were able to acquire carbapenemases and other resistant traits due to their particular preferential for specific plasmids, including IncFII, IncN and IncL/M (Andrade et al., 2011). This observation exemplifies once more the genetic capitalism concept for which resistant organisms tend to be more resistant (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

## CONCLUDING REMARKS

Although in recent years more interest has given to carbapenemases than ESBLs, the CTX-M family still deserve the close attention of researchers and constitute a paradigm of the emergence and spread of a resistant mechanism. Previous descriptions of hierarchical structures that include the *bla*<sub>CTX-M</sub> genes delineated the importance of these structures for maintenance and spread of these genes. Comparison of *bla*<sub>CTX-M</sub> sequences with sequence databases hypothetically defined that *bla*<sub>CTX-M</sub> genes have their ancestors in different *Kluyvera* spp. Nevertheless, *in silico* analyses also suggest that *bla*<sub>CTX-M</sub> genes might also have circulated among different *Kluyvera* species and that the incorporation to these organisms might well have occurred after these species diverged. In Enterobacteriaceae, the great adaptive success of *bla*<sub>CTX-M</sub> genes has been associated with few surrounding genetic structures, few plasmids and few bacterial clones. Successful associations of these units and co-existence of *bla*<sub>CTX-M</sub> genes with other resistance determinants might have contributed to the extraordinary spread of the CTX-M enzymes deserving an uncontrolled pandemic scenario. Future persistence of *bla*<sub>CTX-M</sub> genes within bacterial communities will be assured by co-selection processes as other resistance genes, including emerging ones such as carbapenemases, are now often present in CTX-M producing organisms. Persistence will also give new opportunities for evolution.

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